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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : A61K 31/17, 31/70

A1

(11) International Publication Number: WO 88/ 04925

(43) International Publication Date: 14 July 1988 (14.07.88)

(21) International Application Number: PCT/US87/03468

(22) International Filing Date: 21 December 1987 (21.12.87)

(31) Priority Application Number: 947,227

(32) Priority Date: 29 December 1986 (29.12.86)

(33) Priority Country: US

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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).

**Published**
*With international search report.*

(54) Title: PROTECTION AGAINST AMINOGLYCOSIDE-INDUCED NEPHROTOXICITY

(57) Abstract

The *in vivo* use of compounds which prevent the generation of, effectively scavenge, or detoxify a reactive oxygen metabolite that mediates a toxic effect of an aminoglycoside. The compounds of the invention can be used to prevent or reduce aminoglycoside-induced renal damage, and include but are not limited to free radical scavengers, iron chelators, oxidizable compounds, enzymes which metabolize reactive oxygen metabolites or their precursors, and biosynthetic precursors thereof.

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# PROTECTION AGAINST AMINOGLYCOSIDE-INDUCED NEPHROTOXICITY

## 1. INTRODUCTION

The present invention is directed to the *in vivo* use of compounds that prevent the generation of, effectively scavenge, or detoxify a reactive oxygen metabolite that mediates a toxic effect of an aminoglycoside. The compounds of the invention include agents which prevent the generation of, effectively scavenge, or detoxify free radicals such as the hydroxyl radical, or their metabolic precursors such as hydrogen peroxide and superoxide radical. In a specific embodiment of the invention, compounds that are hydroxyl radical scavengers can provide protection against the nephrotoxicity of aminoglycosides. In another embodiment of the invention, compounds which are iron chelators can reduce aminoglycoside-induced renal damage.

## 2. BACKGROUND OF THE INVENTION

### 2.1. AMINOGLYCOSIDE ANTIBIOTICS

The aminoglycoside antibiotics (*e.g.*, streptomycin, gentamicin, kanamycin, tobramycin, etc.) are widely used in the treatment of infections caused by gram-negative bacteria. The aminoglycosides (aminoglycosidic aminocyclitols) all contain amino sugars in glycosidic linkage to a hexose (aminocyclitol) nucleus. The hexose is either streptidine (in streptomycin) or 2-deoxystreptamine. Aminoglycoside families are distinguished on the basis of the amino sugars attached to the hexose (Goodman and Gilman, eds., 1980, The Pharmacological Basis of Therapeutics, 6th Ed., Ch. 51, pp. 1162-1199).

The rapid bactericidal action of the aminoglycoside antibiotics occurs by inhibition of protein synthesis in susceptible microorganisms. Some susceptible microorganisms include *Escherichia spp.*, *Haemophilus spp.*, *Listeria spp.*, *Pseudomonas spp.*, *Nocardia spp.*, *Yersinia spp.*, *Klebsiella*

spp., Enterobacter spp., Salmonella spp., Staphyloccocus spp., Streptococcus spp., Mycobacteria spp., Shigella spp., and Serratia spp., to name but a few. Protein synthesis inhibition appears to occur by a direct action on the 30S ribosomal subunit, causing interference with translation, initiation, and misreading of the genetic code (Goodman and Gilman, supra).

By virtue of their amino groups, all the aminoglycosides are cationic at physiological pH, with the degree of cationicity being a function of both the number of amino groups present and their positions within the molecule. The polarity of the aminoglycosides is primarily responsible for the pharmacokinetic properties shared by the members of the group. For instance, these drugs are not adequately absorbed after oral administration, they do not easily penetrate the cerebrospinal fluid, and they are rapidly excreted by the kidney. Also, since they are highly polar, there is little passive diffusion, and they must be actively transported across the cell membrane (Goodman and Gilman, supra). The cationicity also appears to play a critical role in aminoglycoside toxicity.

Serious toxicity is a major limitation to the usefulness of aminoglycosides (reviewed in Humes, H.D. and Weinberg, J.M., 1986, Toxic Nephropathies, in The Kidney, Brenner, B.M. and F.C. Rector, eds., W.B. Saunders Company, Philadelphia, Pa., pp. 1491-1532; see also Goodman and Gilman, supra). Three types of toxicity are often encountered with the use of aminoglycosides: (1) ototoxicity, which can involve both auditory and vestibular functions of the eighth cranial nerve; (2) nephrotoxicity, which is manifest as acute tubular necrosis; and (3) acute toxicity, which can follow intrapleural and intraperitoneal administration and is manifest as a neuromuscular blockade culminating in respiratory distress.

Nephrotoxicity is a major complication of the use of antibiotic aminoglycosides (reviewed in Humes, H.D. and Weinberg, J.M., supra), accounting for 10 to 15% of all cases of acute renal failure. It is initially manifested as enzymuria. As early as 24 hours after a single dose administration of aminoglycoside, activities of brush border membrane enzymes can be detected in the urine, with progressive increases in activity as therapy continues. Proximal tubule transport processes are also impaired, and lead to glycosuria, aminoaciduria, and other Fanconi-like syndrome-associated defects, and tubular proteinuria with $beta_2$-microglobinuria. Renal $K^+$ and $Mg^{++}$ wasting may also occur, leading to overt hypokalemia and hypomagnesemia. Polyuria as a result of vasopressin-resistant urinary concentrating defect also develops early in the course of aminoglycoside nephrotoxicity.

The acute renal failure occurs typically after 5-7 days of treatment and is manifested clinically by progressive increases in blood urea nitrogen and plasma creatinine levels. The most evident histopathological change under light microscopy is proximal tubule necrosis. Tubule ultrastructure shows evidence of prominent cytosegresomes that contain concentric laminated dense membranes, designated as myeloid bodies. The specificity of gentamicin for renal toxicity is apparently related to its preferential accumulation in the renal proximal convoluted tubules (50 to 100 times greater than serum). However, the cause for the observed fall in the glomerular filtration rate is unclear, since histological damage is limited to the proximal tubule cells.

Although gentamicin's effect on biological membranes is presumably critical in the pathogenetic sequence, the exact mechanisms of its nephrotoxicity are unknown. However, aminoglycoside cationicity is presumed to play an important role in its nephrotoxicity. As a result of aminoglycoside

cationicity, acidic phospholipids are the major membrane binding site of the drugs. The affinity of aminoglycosides for membrane acid phospholipids may potentially influence multiple plasma membrane and subcellular membrane processes, because membrane acid phospholipids contribute to membrane structure and permeability, and play an important role in the function of membrane bound enzymes and in hormone-membrane receptor interactions. Some of the observed in vitro effects of aminoglycosides on cellular and subcellular membranes include (Humes and Weinberg, supra): (a) inhibition of the activity of sodium-potassium ATPase, (b) inhibition of the activity of lysosomal phospholipases A and C from renal cortex and of extralysosomal phosphatidylinositol-specific phospholipase C, (c) inhibition of sodium-dependent glucose uptake in isolated renal brush border membranes, (d) inhibition of the ability of anti-diuretic hormone to stimulate adenyl cyclase and thus increase water permeability in isolated toad urinary bladder, (e) stabilization of lysosomes at low aminoglycoside concentrations, and increase in lysosomal lability at high aminoglycoside concentrations, and (f) mitochondrial swelling and alterations in mitochondrial respiratory function. Observed in vivo effects of aminoglycosides on subcellular systems include (Humes and Weinberg, supra): (a) lysosomal alterations such as inhibition of lysosomal sphingomyelinase and phospholipase A, and increases in both lysosomal and extralysosomal phosphatidylinositol, (b) tubule transport abnormalities in early stages of nephrotoxicity, as manifested by glycosuria and potassium and magnesium wasting, (c) abnormalities in mitochrondria functioning, and (d) a decline in superficial nephron glomerular filtration rate, primarily due to reduction in the glomerular capillary ultrafiltration coefficient (and probably secondary to intrarenal angiotensin II generation).

There is thus a large body of information on the in vitro and in vivo effects of aminoglycosides. This has led to several hypotheses to explain the pathogenesis of aminoglycoside-induced renal failure (reviewed in Humes and Weinberg, supra), which include disruptions of lysosomal, mitochondrial, and plasma membrane structure and function. However which subcellular or cellular component of the cell is most critical in the development of aminoglycoside cell injury is not known.

Studies preventing a potentially critical biological effect of an aminoglycoside have not necessarily led to amelioration of nephrotoxicity. For example, it has been unequivocally shown that increased lipid peroxidation occurs in the renal cortex of rats injected with gentamicin (Ramsammy, L.S., et al., 1985, Biochem. Pharmac. 34:3895-3900). This raised the possibility that lipid peroxidation may participate in the pathogenesis of aminoglycoside nephrotoxicity. The hypothesis that lipid peroxidation is linked causally to the pathogenesis of aminoglycoside nephrotoxicity was tested by determining whether administration of diphenyl-phenylenediamine (DPPD) would inhibit lipid peroxidation and thus ameliorate gentamicin-induced renal failure (Ramsammy, L.S., et al., 1986, J. Pharm. Exp. Ther. 238:83). Gentamicin increased the lipid peroxidation product, malondialdehyde in rat renal cortex. However, while concurrent treatment with DPPD inhibited the lipid peroxidation, it did not prevent either the functional or the histological renal damage caused by gentamicin treatment. This study thus illustrates that the fact that aminoglycosides influence some biological processes, either in vitro or in vivo, does not necessarily predict the importance of such influence in the pathogenesis of acute renal failure, i.e. preventing specific biological effects of aminoglycosides does not necessarily lead to prevention of acute renal failure.

Other studies have looked at the effects of additional compounds on aminoglycoside activity and toxicity. PCT International Application Number PCT/US84/00855, Publication Number W085/05030 (published November 21, 1985) discloses a method for reducing aminoglycoside toxicity by mixing the aminoglycoside, before administration, with ligands (lipids or lipid head groups) which subsequently prevent or reduce binding of the drug to its endogenous toxicity receptor. Polyamino acids such as polyasparagine and polyaspartic acid have been shown to inhibit gentamicin binding to renal brush border membrane vesicles in vitro and to inhibit gentamicin and amikacin nephrotoxicity in rats (Williams, P.D., et al., 1986, J. Pharm. Exp. Therap. 237:919). Side effects of aminoglycoside antibiotics, renal toxicity, and 8th nerve toxicity can be reduced by administering 2,5-di-O-acetyl-D-gluco-saccharo-1,4: 6,3-dilactone (U.S. Patent No. 3,928,583 by Furuno et al.) or certain other glucosaccharic acids or their metal salts (U.S. Patent No. 3,962,429 by Furuno et al.). Oxygen radical activation of aminoglycosides, for example, by use of superoxide and/or hydroxyl radicals, appears to decrease the drugs' polarity, thus enhancing their cell membrane penetration and increasing bactericidal effectiveness (European Patent Application Number 83402394.7, Publication Number 0 134 372, published March 20, 1985). The role of the iron chelator deferoxamine was investigated in vitro with regard to its synergistic effect on the antibacterial action of aminoglycosides (van Asbeck, B.S., et al., 1983, Eur. J. Clin. Microbiol. 2:432-438). 2,3-dihydroxybenzoic acid or dimethyl sulfoxide has been administered to gentamicin-treated rats which had intra-abdominal sepsis, in order to determine the compound's effect on mortality due to severe sepsis (Pearce,

R.A., et al., 1985, Arch. Surg. 120:937). Only 2,3-dihydroxybenzoic acid, and not dimethyl sulfoxide, increased survival time.

## 2.2. REACTIVE OXYGEN METABOLITES

The complete reduction of oxygen by the univalent pathway results in the formation of superoxide anion radical, hydrogen peroxide and hydroxyl radical (OH·) as intermediates (Fridovich, I., 1976, Oxygen radicals, hydrogen peroxide and oxygen toxicity, in Free Radicals in Biology, Vol. I, Academic Press, pp. 239-278; Mastro, R.F., 1980, Acta. Physiol. Scand. Supp. 92: 153-168). These intermediates are too reactive to be tolerated in living tissue, and a variety of enzymatic mechanisms which can bypass the electron spin restriction of oxygen and accomplish the divalent and tetravalent reduction of oxygen to water have evolved. Thus, most of the oxygen consumed by respiring cells is utilized by cytochrome oxidase which reduces oxygen to water without releasing either superoxide or hydrogen peroxide (Fridovich, I., 1976, supra). Despite this, in respiring cells at least some reduction of oxygen occurs via the univalent path. In in vitro studies, the ability of microsomes and mitochondria to generate superoxide and hydrogen peroxide (Chance, B., et al., 1979, Physiol. Rev. 59:527-605; Forman, H.J., and Boveris, A., 1982, Superoxide radical and hydrogen peroxide in mitochondria, in Free Radicals in Biology, Academic Press, pp. 65-90) has been demonstrated. Agents which affect mitochondrial respiration have been shown to enhance hydrogen peroxide generation (Forman and Boveris, supra; Doroshow, J.H. and Davies, K.J.A., 1986, J. Biol. Chem. 261:3068-3074). We have previously shown that gentamicin increases the production of hydrogen peroxide by renal cortical mitochondria (Walker, P.D., et al., 1985, Gentamicin induced generation of hydrogen peroxide by renal mitochondria (mito),

Abstract, American Society of Nephrology Meeting, December, 1984, Kidney International 27:238; Walker, P.D., et al., 1986, Reactive oxygen metabolites and lipid peroxidation in gentamicin nephrotoxicity, Abstract, International Academy of Pathology Meeting, March 1986, Lab. Invest. 54:67A). Most, if not all of the hydrogen peroxide generated by mitochondria is derived from superoxide anion (Forman, H.J. and Boveris, A., supra).

The enzymatic defenses against superoxide and hydrogen peroxide include superoxide dismutase, catalase, and glutathione peroxidase. Superoxide dismutase converts the superoxide radical into hydrogen peroxide and molecular oxygen:

$$2O_2^- + 2H^+ \dashrightarrow H_2O_2 + O_2$$

Two superoxide dismutases have been identified in mammalian tissues, a cytoplasmic copper-zinc and a mitochondrial manganese-dependent enzyme (reviewed in Fantone, J.C. and Ward, P.A., 1982, Amer. J. Pathology 107:397-416; Fantone, J.C. and Ward, P.A., 1985, Human Pathology 16(10):973-978; Fridovich, I., 1979, Superoxide dismutase: defense against endogenous superoxide radical, in Oxygen free radicals and tissue damage, Ciba Symposium 65:77-85). The enzymatic mechanisms for cellular detoxification of hydrogen peroxide are catalase and glutathione peroxidase (reviewed in Fantone, J.C. and Ward, P.A., 1982, supra; Fridovich, I., 1976, supra; Maestro, R.F., 1980, Acta. Physiol. Scand. Supp. 92:153-168; Chance, B., et al., 1979, Physiol. Rev. 59:527-605). Catalase, a cytoplasmic heme-enzyme, catalyses the divalent reduction of hydrogen peroxide to water:

$$2H_2O_2 \dashrightarrow 2H_2O + O_2$$

Glutathione peroxidase, a selenium-dependent enzyme, is effective at low concentrations of hydrogen peroxide and can also act upon lipid hydroperoxides, thus countering the toxicity of a wide range of peroxides (Lawrence, R.A. and Burk, F.R., 1976, Biochem. Biophys. Res. Commun. 71:952-958). Recently, a selenium-independent glutathione peroxidase activity which can detoxify organic peroxides but not metabolize hydrogen peroxide has been identified (Lawrence, R.A. and Burk, R.F., 1978, J. Nutr. 108:211-215).

In addition to the enzymatic mechanisms, cellular detoxification also appears to be mediated by reduced glutathione (GSH). GSH, a tripeptide which occurs in high concentrations in virtually all mammalian cells, appears to function in the protection of cells against the effects of free radicals and reactive oxygen intermediates (e.g. peroxides) (Meister, A., 1983, Science, 22:472-478; Meister, A. and Anderson, M.E., 1983, Ann. Rev. Biochem. 52:711-60; Meister, A., 1984, Hepatology 4(4):739-742; Andreoli, S.P., et al., 1986, J. Lab. Clin. Med. 108(3):190-198; Jensen, G.L. and Meister, A., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4714-4717; Dethmers, J.K. and Meister, A., 1981, Proc. Natl. Acad. Sci. U.S.A. 78(12):7492-7496; Arrick, B.A., et al., 1982, J. Biol. Chem. 257(3):1231-1237).

When the generation of superoxide and hydrogen peroxide is enhanced, superoxide and hydrogen peroxide may not only be directly cytotoxic, but, in addition, may interact (with iron as catalyst) by the Haber Weiss reaction to generate the hydroxyl radical (Hoe, S., et al., 1982, Chem.-Biol. Interactions 41:7501; Aust, C.D., et al., 1985, J. Free Radicals Biology & Medicine 1:3-25):

$$Fe^{3} + O_2^- \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2-} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^-$$

Several studies have shown that agents that enhance the generation of hydrogen peroxide and superoxide anion by mitochondria also enhance the generation of hydroxyl radical (Doroshow and Davies, supra; Komiyama, T., et al., 1982, Biochem. Pharm. 31(22):3651-3656).

The cytotoxicity of reactive oxygen metabolites, including free radical species (e.g. superoxide and hydroxyl radicals) and other oxygen metabolites (e.g. hydrogen peroxide, hypochlorous acid) is well documented (Fantone, J.C. and Ward, P.A., 1982, Am. J. Pathol. 107:397-418; Fantone, J.C. and Ward, P.A., 1985, Hum. Pathol. 16:973-978; Weiss, S.J. and LoBuglio, A.F., 1982, Lab. Invest. 47(1):5-18). In particular, recent in vivo studies have demonstrated the protective effect of hydroxyl radical scavengers and/or iron chelators (presumably by preventing the generation of hydroxyl radical by the iron-catalysed Haber Weiss reaction) in several models of tissue injury (Ward, P.A., et al., 1985, J. Clin. Invest. 76:517-523; Ward, P.A., et al., 1983, J. Clin. Invest. 72:789-801; Fox, R.B., 1984, J. Clin. Invest. 74:1456-1464; Fligiel, S.E.G., et al., 1984, A.J.P. 115(3):375-382; Johnson, K.J., et al., 1986, Lab. Invest. 54(5):499-506; Till, G.O., et al., 1985, A.J.P. 119(3):376-384). In addition, both in vitro and in vivo studies have suggested a role of glutathione in protecting against adriamycin (an anthracycline antibiotic) cardiotoxicity, presumably by its detoxification of oxidative free radicals (Olson, R.D., et al., 1981, Life Sciences 29:1393-1401; Yoda, Y., 1986, Cancer Res. 46:251). Some

limited studies have examined the role of reactive oxygen metabolites in renal disease. We have shown that reactive oxygen metabolites affect several biological processes potentially important in glomerular diseases (Shah, S.V., 1984, J. Clin. Invest. 74:393-401), and their role in neutrophil-mediated glomerular diseases has been demonstrated by others (Rehan, A., et al., 1984, Lab. Invest. 51:396-403; Rehan, A., et al., 1985, Kidney Intl. 27:503-511; Rehan, A., et al., 1986, Am. J. Physiol. 123(1):57-66). In addition, reactive oxygen metobolites have been postulated to be important in ischemic acute renal failure (Paller, M.S., et al., 1984, J. Clin. Invest. 74: 1156-1164). However, the role of reactive oxygen metabolites in aminoglycoside nephrotoxicity has not been previously examined.

## 3. SUMMARY OF THE INVENTION

The present invention is directed to the in vivo use of compounds, termed hereinafter "protective agents", which prevent the generation of, effectively scavenge, or detoxify a reactive oxygen metabolite (ROM) that mediates a toxic effect of an aminoglycoside. The protective agents of the invention include but are not limited to free radical scavengers, iron chelators, and enzymes which metabolize reactive oxygen metabolites, converting them to less toxic states and/or preventing the production of other toxic species. The protective agents also include oxidizable compounds which effectively detoxify the ROMs, exerting a protective effect by undergoing oxidation in lieu of important cellular components. Another group of protective agents includes any compounds (e.g. biosynthetic precursors) which increase the effective in vivo concentrations of endogenous protective agents.

The invention is based, in part, on the discovery that the nephrotoxic effects of aminoglycosides in vivo are mediated by ROMs. The protective agents can be used therapeutically, in accordance with the present invention, before, during, or after aminoglycoside administration to prevent or reduce aminoglycoside-induced nephrotoxicity. In specific embodiments, hydroxyl radical scavengers or iron-chelators can be used to protect against renal damage. In another aspect of the invention, enzymes such as catalase and/or superoxide dismutase can be used to convert the reactive metabolites $H_2O_2$ and $O_2^-$ to less harmful products and to prevent the generation of other toxic metabolites. In particular embodiments, the iron-chelator deferoxamine, the hydroxyl radical scavenger dimethylthiourea, or glutathione biosynthetic precursors can be administered to protect against antibiotic aminoglycoside-induced nephrotoxicity.

## 3.1. DEFINITIONS

The following terms and abbreviations will have the meanings indicated:

Protective agent = A compound that prevents the generation of, effectively scavenges, or detoxifies an aminoglycoside-induced reactive oxygen metabolite which mediates a toxic effect.

OC = Oxidizable compound. A protective agent which detoxifies a reactive oxygen metabolite by undergoing oxidation by the reactive oxygen metabolite, in lieu of and preventing the detrimental oxidation of other cellular components.

BUN = blood urea nitrogen.

DMSO = dimethyl sulfoxide.

DMTU = dimethylthiourea

ROM = reactive oxygen metabolite.

## 4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1A depicts the protective effect of hydroxyl radical scavengers dimethylthiourea (DMTU) and dimethyl sulfoxide (DMSO) on gentamicin (GENT)-induced acute renal failure, as measured by blood urea nitrogen (mg/dl). Saline represents a control group of saline-treated rats. N equals the number of animals in each group.

Figure 1B depicts the protective effect of hydroxyl radical scavengers dimethylthiourea (DMTU) and dimethyl sulfoxide (DMSO) on gentamicin (GENT)-induced acute renal failure, as measured by plasma creatinine levels (mg/dl). Saline represents a control group of saline-treated rats. N equals the number of animals in each group.

Figure 2A depicts the protective effect of the hydroxyl radical scavenger sodium benzoate (BENZOATE) and the iron chelator deferoxamine (DFO) on gentamicin (GENT)-induced acute renal failure, as measured by blood urea nitrogen (mg/dl). Saline represents a control group of saline-treated rats. N equals the number of animals in each group.

Figure 2B depicts the protective effect of the hydroxyl radical scavenger sodium benzoate (BENZOATE) and the iron chelator deferoxamine (DFO) on gentamicin (GENT)-induced acute renal failure, as measured by plasma creatinine levels (mg/dl). Saline represents a control group of saline-treated rats. N equals the number of animals in each group.

Figure 3 depicts the protective effect of the iron chelator 2,3-dihydroxybenzoic acid (DHB) on gentamicin (GENT)-induced acute renal failure, as measured by blood urea nitrogen (mg/dl). Saline represents a control group of saline-treated rats.

Figure 4A is a light microscopic section of kidneys from rats receiving gentamicin alone, showing severe tubular epithelial necrosis with sloughing of the lining epithelium and luminal debris. Magnification is X 200.

Figure 4B is a light microscopic section of kidneys from rats receiving gentamicin plus deferoxamine showing essentially no pathologic abnormalities. Magnification is X 200.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of compounds *in vivo* that prevent the generation of, effectively scavenge, or detoxify a reactive oxygen metabolite (ROM) that mediates a toxic effect of an aminoglycoside. The invention is based, in part, on the discovery that the nephrotoxic effects of aminoglycosides *in vivo* are mediated by ROMs. The compounds of the invention act by preventing the production of, by removing, or by preventing the detrimental reaction with cellular components of hydroxyl radicals, superoxide radicals, hydrogen peroxide, and other ROMS. These compounds shall be termed hereinafter "protective agents".

### 5.1. PROTECTIVE AGENTS

The protective agents of the present invention are compounds that can be used *in vivo* to prevent toxic side effects such as renal damage caused by aminoglycosides. The protective agents exert their effect by preventing the generation of, by effectively scavenging, or by detoxifying ROMs, and include but are not limited to free radical and other

ROM scavengers, iron chelating agents, and compounds (e.g. biosynthetic precursors) which increase the effective in vivo concentrations of endogenous protective agents. The scavengers of ROMs which may be used in the practice of the present invention include but are not limited to scavengers of hydroxyl radicals, superoxide radicals, hydrogen peroxide, and singlet oxygen. The hydroxyl radical scavengers of the present ivnention include but are not limited to dimethylthiourea, dimethyl sulfoxide, and sodium benzoate. The protective agents also include but are not limited to enzymes (e.g. superoxide dismutase, catalase, and glutathione peroxidase) which convert ROMs to less toxic states or metabolize ROMs (e.g. $O_2^-$ and $H_2O_2$) thus preventing the further generation of other ROMs. Another category of protective agents includes nonenzymatic, oxidizable compounds (termed hereinafter OCs) which effectively detoxify the ROMs by undergoing oxidation in lieu of important cellular components. Such OCs include but are not limited to thiols, e.g. glutathione. Because thiols are easily oxidized, they may be preferentially oxidized by the reactive oxygen metabolites, thereby protecting the tissues from oxidative damage. Molecules which are metabolic precursors of OCs can be administered in order to increase effective endogenous OC concentrations in vivo. For example, biosynthetic precursors of reduced glutathione can be used which include but are not limited to gamma-glutamylcysteine, gamma-glutamylcysteine disulfide, and gamma-glutamylcystine (Anderson, M.E. and Meister, E., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:707-711). The iron chelators of the present invention include compounds that bind iron which is necessary for the generation of toxic free radicals or their precursors, thus preventing such generation. Metabolic precursors of free radicals which the protective agents of the present invention can convert to less harmful products, include but are not limited to hydrogen

peroxide and superoxide radical, which, if not converted by protective agents, can react to produce hydroxyl radicals. The ROM scavengers, iron chelators, and enzymes of the invention are molecules that can effect their protective function in vivo at the appropriate site of ROM generation or accumulation, without significant toxic effects. The protective agents for use in the present invention include but are not limited to the scavengers, OCs, metabolic precursors, iron chelators, and enzymes of Table I, infra.

TABLE I

AGENTS WHICH CAN BE USED TO PROTECT AGAINST TOXIC EFFECTS OF AMINOGLYCOSIDES

I. ROM SCAVENGERS, OCs, OR METABOLIC PRECURSORS THEREOF[1]

- dimethylthiourea
- dimethyl sulfoxide
- sodium benzoate
- tryptophan
- azide
- dabco
- histidine
- mercaptoalkylamines
  - 2-mercaptoethylamine and derivatives
  - glutathione
  - 3-aminopropanethiol (3-mercaptopropylamine), 2-aminopropanethiol
  - 1-amino-2-propanethiol
  - DL-trans-2-aminocyclohexanethiol and derivatives
  - 2-(3-aminopropylamino)ethanephosphorothioic acid (WR 2721)
- N-(2-mercaptopropionyl)-glycine
  - gamma-glutamylcysteine
- gamma-glutamylcysteine disulfide
- gamma-glutamylcystine
- cysteine
- cysteine derivatives:
  - cysteine methyl ester hydrochloride
  - cysteine ethyl ester hydrochloride
  - cysteine propyl ester hydrochloride
  - cysteine isopropyl ester hydrochloride

cysteine butyl ester hydrochloride
cysteine isobutyl ester hydrochloride
cysteine isoamyl ester hydrochloride
rutosidyl-2'-methylenecysteine
2,3-dimercaptopropanesulfonate (Unithiol)
Cleland's reagent and derivatives
bis(2-aminoethyl) disulfide (cystamine)
thioctic acid
2-aminoethyl 2-aminoethanethiolsulfonate
organic thiosulfates (Bunte salts)
2-aminoethanethiosulfuric acid
2-aminopropane-1-thiosulfuric acid
N-alkylated-2-aminoethanethiosulfuric acids
N-(4-phenylbutyl) aminoethanesulfuric acid and derivatives
2-guanidinoethanethiosulfuric acid
sodium cysteinethiosulfate
phosphorothioates and derivatives
sodium 2-aminoethanephosphorothioate
2-guanidinoethanephosphorothioate
3-guanidinopropanephosphorothioate
Other thioureas
thiourea
methylthiourea
ethylenethiourea
methylthiopseudourea
ethylthiopseudourea
α,ω-bis(thiopseudoureas)
5-ethylisothiuronium ethyl phosphine
2-aminoethylisothiuronium bromide hydrobromide (AET)
aminoethylisothiuronium adenine triphosphate (Adeturon)

bis(2-guanidinoethyl) disulfide (GED)
2-aminobutylthiopseudourea dihydrobromide
thiazolines
thiazolidines and derivatives
gallic acid derivatives
sodium gallate
propyl gallate
p-aminoacetophenone
p-aminopropiophenone (PAPP)

II. IRON CHELATORS
deferoxamine (deferoxamine B mesylate)
2, 3-dihydroxybenzoic acid
diethylenetriaminepentaacetic acid (DETAPAC, DTPA)
apolactoferrin (lactoferrin)

III. ENZYMES
superoxide dismutase
catalase
glutathione peroxidase

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[1]For a discussion of some of these compounds, see Kirk-Othmer, Encyclopedia of Chemical Technology, 3rd Ed., Vol. 19, 1982, John Wiley & Sons, New York, pp. 801-832.

## 5.2. THERAPEUTIC USES OF PROTECTIVE AGENTS

The protective agents of the present invention can be used to protect against the toxic side effects of any aminoglycoside whose toxic effect is mediated by a reactive oxygen metabolite. The protective agents may be administered prior to, concurrently with, or after the administration of aminoglycosides, in order to prevent or reduce toxicity. The protective agents can be administered by any of a variety of routes, including but not limited to intraperitoneal, intravenous, subcutaneous, oral, intranasal, intramuscular, etc. The protective agents can be delivered in various formulations. They can be incorporated into or on liposomes, modified by conjugation to polymers or carrier molecules, etc. Such formulations can be used to enhance the desired localization, delivery, or cellular penetration. For example, superoxide dismutase or catalase can be encapsulated in liposomes to enhance their intracellular delivery.

When an aminoglycoside is administered therapeutically, the protective agents can be included in the therapeutic regimen to prevent undesired side effects. Such therapeutic uses of aminoglycosides include but are not limited to the use of antibiotic aminoglycosides in the treatment of bacterial infections. Protective agents can be used to reduce toxicity when aminoglycosides are employed to treat septicemia, to prevent the *in vivo* formation of certain bacterial metabolic products, or to prevent the *in vivo* bacterial degradation of certain compounds, etc. In particular embodiments, free radical scavengers, iron chelators, superoxide dismutase, or catalase can be administered both concurrently and after the administration of antibiotic aminoglycosides. Aminoglycosides

whose detrimental side effects may be avoided or reduced in accordance with the present invention include but are not limited to those listed in Table II, infra.

TABLE II

AMINOGLYCOSIDES WHOSE TOXIC EFFECTS MAY BE REDUCED OR PREVENTED BY ADMINISTRATION OF PROTECTIVE AGENTS

Neomycin A
Neomycin B
Neomycin C
Paramomycin I
Paramomycin II
Ribostamycin
Lividomycin
Kanamycin A
Kanamycin B
Kanamycin C
Amikacin
Dibekacin
Butakacin
Tobramycin
Gentamicin B
Gentamicin $C_1$
Gentamicin $C_{1a}$
Gentamicin $C_2$
Gentamicin $C_{2b}$
Gentamicin $X_2$
Gentamicin J1 20A
Gentamicin derivatives:
- Sch 20278
- Sch 21420
- Sch 23722
- Sch 24443

Sch 21211
Sch 21768
Sch 23200
Sch 23456
2',3'-dideoxygentamicin B
Streptomycin
Dihydrostreptomycin
Sisomicin
5-Epi-sisomicin
G-52
Verdamicin
Netilmicin
Sch 21562
Sch 27082
Sch 22591
Sch 27082
Sch 27598
Framycetin
Apramycin
Fortimicin A
Fortimicin B
Butikacin
Propikacin
5"-amino-5"-deoxybutirosin A

5.2.1. PROTECTION AGAINST RENAL DAMAGE

One of the detrimental side effects of aminoglycoside administration is nephrotoxicity. The protective agents of the present invention can be used before, during, or after administration of aminoglycosides to protect against renal damage resulting from reactive oxygen metabolite production. Toxic effects on the kidney caused by aminoglycoside administration, that can be reduced or prevented by the protective agents include, for example, acute tubular necrosis and renal failure (see Section 6., infra). In particular embodiments of the present invention, protective agents such as dimethylthiourea, sodium benzoate, dimethyl sulfoxide, deferoxamine, or 2,3-dihydroxybenzoic acid can be used to reduce the renal damage induced by the nephrotoxic compounds tobramycin or gentamicin. In one example of this embodiment, the iron-chelator deferoxamine can be formulated with gentamicin and a pharmaceutical carrier, and administered intramuscularly for the prevention of gentamicin-induced nephrotoxicity. In another particular embodiment, glutathione biosynthetic precursors can be used to increase the renal concentration of the endogenous OC glutathione. Studies have shown that the synthetic precursors gamma-glutamyl cysteine and gamma-glutamylcystine, when administered subcutaneously, will increase levels of glutathionine in the kidney (Anderson, M.E. and Meister, A., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:707-711). Careful monitoring of the patient's renal functioning can be done, by measurement of basal urine nitrogen (BUN), plasma creatinine levels, trough concentration of amino-glycoside, or any other standard techniques.

## 6. EXAMPLE: PREVENTION OF GENTAMICIN-INDUCED ACUTE RENAL FAILURE IN RATS

The experiments detailed in the example sections infra demonstrate that treatment with compounds which prevent the generation of or effectively scavenge hydroxyl radicals effectively protects against gentamicin-induced acute renal failure in rats.

### 6.1. GENERAL PROCEDURES

#### 6.1.1. ANIMALS

Adult male Sprague-Dawley rats weighing 200-250 g and having free access to water and standard rat chow (1.00% calcium, 0.21% magnesium, 0.40% sodium, 1.10% potassium) were used in these experiments. The rats received daily subcutaneous injections of either 1 ml of sterile, isotonic saline or gentamicin (100 mg/Kg/day) for 8 consecutive days (Humes, H.D., et al., 1984, J. Clin. Invest. 73:134-147). Twenty-four hours after the last injection, the rats were sacrificed, plasma was obtained for the measurement of BUN (blood urea nitrogen) and/or creatinine, and kidneys were isolated for histological examination and determination of gentamicin levels.

#### 6.1.2. INTERVENTIONAL THERAPY

The effect of several hydroxyl radical scavengers and of an iron chelator on gentamicin-induced acute renal failure was examined. Dimethylthiourea (DMTU) was administered in a dose of 500 mg/Kg intraperitoneally (IP) just prior to the first gentamicin injection followed by 125 mg/Kg IP twice a day. The other hydroxyl radical scavengers used were dimethyl sulfoxide (DMSO), which was administered at 4 gm/Kg (Lotan, D., et al., 1984, Kidney Intl. 25: 778-788) twice a day intraperitoneally,

and sodium benzoate, at a dosage of 150 mg/Kg IP twice a day. The iron chelator deferoxamine B mesylate (Desferal, Ciba-Geigy Corp., Summit N.J.) was administered intravenously in doses of 20 mg/rat just prior to the first gentamicin injection. At the same time, deferoxamine was administered via an osmotic pump (type 2ML 2: ALZA Corp., Palo Alto, CA) that was implanted subcutaneously. The drug was reconstituted in water at a concentration of 175 mg/ml, and the pumps (with a 2 ml capacity) delivered 20 mg deferoxamine per rat at a continuous rate of 5 ul/hr. Previous studies have shown that constant plasma levels of the drug are maintained when the deferoxamine is administered by this route (Bowern, N., et al., 1984, J. Exp. Med. 160:1532-1543).

6.1.3. QUANTITATIVE ASSAYS FOR IN VIVO STUDIES

Urea nitrogen and creatinine were measured in plasma samples using the Beckman BUN Analyzer 2 and the Beckman Creatinine Analyzer.

For the gentamicin assay, one part renal cortical homogenate was diluted with nine parts 0.15% Triton X-100 in distilled water. A further dilution was then made to produce a protein concentration of 1 mg/ml and the concentration of gentamicin was determined using a standard enzymatic radiochemical assay (Smith, D.H., et al., 1972, Medical Intelligence 286 (11):583-586). Briefly, $^{14}C$-labeled acetyl-coenzyme A was incubated with sample containing gentamicin and gentamicin acetyl transferase for ten minutes, allowing complete conversion of gentamicin to acetyl-gentamicin. The reaction liquid was then pipetted onto a phosphocellulose disc to which acetyl-gentamicin binds very tightly. After washing to remove unreacted acetyl-coenzyme A, the bound radioactivity was counted in a liquid scintillation counter. The validity of the assay was assessed by measurement of gentamicin in kidney

homogenates from the non-gentamicin treated rats, and a kidney homogenate from a non-gentamicin treated rat to which gentamicin was added in concentrations of 2, 5, 10, 15, and 20 ug/ml.

6.1.4. HISTOLOGICAL EXAMINATION OF THE KIDNEY

The kidney was sectioned and a portion was fixed in either 10% formalin (for light microscopy) or 3% glutaraldehyde (for electron microscopy). Tissues to be used for light microscopy were dehydrated and embedded in glycol methacrylate. Sections were cut at 2 microns and stained with periodic acid-Schiff (PAS) reagent. The slides were coded and examined without knowledge of the treatment protocol. Tissues for electron microscopy were post-fixed in 1% osmium tetroxide for one hour, dehydrated and embedded in maraglas. Silver sections were obtained, stained with lead citrate and uranyl acetate, and examined in a Phillips 300 electron microscope.

A light microscopy semiquantitative analysis of the kidney sections was performed using the technique of Houghton et al. (Houghton, D.C., et al., 1978, Am. J. Pathol. 93:137-152). The changes seen were limited to the tubulointerstitial areas and were graded as follows: 0 = normal; 1 = Areas of focal granulovacuolar epithelial cell degeneration and granular debris in tubular lumina with or without evidence of tubular epithelial cell desquamation in small foci (less than 1% of total tubule population involved by desquamation); 2 = Tubular epithelial necrosis and desquamation easily seen but involving less than half of cortical tubules; 3 = More than half of proximal tubules showing desquamation and necrosis but uninvolved tubules easily found; 4 = Complete or almost complete proximal tubular necrosis. In addition to grading the histological changes, the presence or absence of cytoplasmic

PAS-positive bodies (confirmed to be cytosegresomes by electron microscopy) in the proximal tubule epithelial cells was also recorded.

6.1.5. IN VITRO STUDIES

We studied the in vitro effect of dimethylthiourea (DMTU) (10 mM) and deferoxamine (1 mg/ml) on gentamicin-enhanced generation of hydrogen peroxide by renal cortical mitochondria. Mitochondria were isolated essentially as described by Johnson and Lardy (1967, in Methods in Enzymology, Vol. 10, Estabrook, R.W. and M.E. Pullman, eds., Academic Press, New York, pp. 94-96). The isolation medium contained 0.27 M sucrose, 1 mM EGTA, 5 mM Tris-HCl, pH 7.4. Mitochondria were sedimented by centrifugation at 600 x g for 10 minutes, followed by centrifugation of the resulting supernatant at 10,000 x g for 10 minutes. The mitochondrial pellet was resuspended in 0.25 M sucrose and centrifuged at 10,000 x g for 10 minutes. The final pellet was suspended in 0.25 M sucrose to give a protein concentration of about 10 mg protein/ml. Only mitochondria that had a respiratory control index (state 3/state 4 respiration) of greater than 2.5 were used for experiments. Hydrogen peroxide production was measured using the scopoletin method (Boveris, A., et al., 1973, Anal. Biochem. 80:145-158). 0.5 mg of mitochondrial protein was added to the reaction mixture containing 150 mM KCl, 10 mM Tris-phosphate, 5 mM Tris-HCl, pH 7.4, and 0.1 ml of horseradish peroxidase (400 ug/ml) in a total volume of 3 ml. Scopoletin was added to a final concentration of 5 nM, and the 100% baseline fluorescence was set in a Farrand System 3 Spectrofluorometer (Farrand Optical Co., Valhalla, New York). The decrease in fluorescence (excitation wavelength 385 nm and emission wavelength 460 nm) was first recorded after the addition of substrate (10 mM sodium succinate, baseline

values), and then after gentamicin (4 mM) was added to the same reaction mixture. The effect of DMTU (10 mM) and deferoxamine (1 mg/ml) added prior to the addition of gentamicin, on gentamicin-stimulated production of hydrogen peroxide was examined.

### 6.2. AN HYDROXYL RADICAL SCAVENGER OR AN IRON CHELATOR PROTECT AGAINST GENTAMICIN-INDUCED RENAL DAMAGE

In our preliminary studies, we examined the time course of the effect of daily subcutaneous injection of gentamicin alone (100 mg/Kg) (Humes, H.D., et al., 1984, J. Clin. Invest. 73:134-147) on renal function (measured by the BUN, blood urea nitrogen, concentration 24 hours after the last injection). A marked increase in the BUN was noted after eight injections in all the gentamicin treated rats. Based on this data, we examined the effect of various interventions on BUN and creatinine levels after eight injections of gentamicin.

#### 6.2.1. DIMETHYLTHIOUREA OR DEFEROXAMINE HAS A PROTECTIVE EFFECT AGAINST GENTAMICIN-INDUCED ACUTE RENAL FAILURE

We examined the effect of dimethylthiourea (DMTU) and of deferoxamine on gentamicin-induced acute renal failure. DMTU *in vitro* acts as an hydroxyl radical scavenger (Fox, R.B., 1984, J. Clin. Invest. 74:1456-1464), and deferoxamine mesylate has been shown to block the generation of hydroxyl radical (Hoe, S., et al., 1982, Chem.-Biol. Interactions, 41:75-81). In addition, *in vivo*, DMTU (administered intraperitoneally) has been shown to achieve concentrations sufficient to scavenge hydroxyl radical with a half-life of 34 hours in rats (Fox, R.B., *supra*).

Rats treated with gentamicin for eight days had a marked increase in BUN (215±30 mg/dl, n=8) compared to saline treated controls (BUN: 16±1 mg/dl, n=8). In contrast, rats treated

with gentamicin and DMTU had significantly lower BUN values (BUN: 48±17 mg/dl, n=8 p 0.0001). Similarly, deferoxamine afforded a marked protective effect (BUN: 30±7 mg/dl, n=8 p 0.0001) against gentamicin-induced acute renal failure.

We considered the possibility that the interventional agents might have caused the gentamicin nephrotoxicity to be manifested at an earlier time point, and that the BUN were lower after eight injections because these rats were in the recovery phase. We therfore examined the effect of deferoxamine or rats receiving six gentamicin injections. In the deferoxamine-treated animals, the BUN was significantly lower (22±2 mg/dl, n=6 p 0.0001) compared to the BUN in rats treated with gentamicin alone (80±5 mg/dl, n=6).

In order to demonstrate that the protective effect of DMTU or deferoxamine was unrelated to gentamicin uptake by the renal tissue, we measured gentamicin levels in the kidney cortices. The values obtained for renal cortical gentamicin concentrations were as follows: gentamicin alone, 14±0.7 ug/mg, n=8 (similar to results obtained by others); gentamicin plus DMTU, 19±1.9 ug/mg; and gentamicin plus deferoxamine, 20±1.5 ug/mg. 1 These results indicated that the protective effect of these agents was unrelated to the uptake of gentamicin by renal cortical tissue.

We also investigated whether the effect of DMTU or deferoxamine might be related to some direct interference with the ability of renal cortical mitochondria to respond to gentamicin. We examined, in vitro, the effect of DMTU or deferoxamine on gentamicin-enhanced generation of hydrogen peroxide by renal cortical mitochondria. DMTU (10 mM) and deferoxamine (1 mg/ml) had no significant effect on gentamicin-enhanced generation of hydrogen peroxide. DMTU (Varani, J., et al., 1985, Lab. Invest. 53(6):656-663; Fox, R.B., 1984, J. Clin. Invest. 74:1456-1464) and deferoxamine

(Varani, J., supra; Ward, P.A., et al., 1983, J. Clin. Invest. 72:789-801) have been similarly shown not to affect generation of superoxide and/or hydrogen peroxide by neutrophils. Taken together, these results demonstrate a significant protective effect of DMTU or deferoxamine on gentamicin-induced acute renal failure, and show that this protective effect is not due to an alteration in the mitochondrial response to gentamicin or in the uptake of gentamicin by renal cortical tissue.

### 6.2.2. DIMETHYLTHIOUREA OR DIMETHYL SULFOXIDE HAVE A PROTECTIVE EFFECT AGAINST GENTAMICIN-INDUCED ACUTE RENAL FAILURE

In a second experiment, we examined the effect of DMTU again and a second hydroxyl radical scavenger, dimethyl sulfoxide (DMSO) (Repine, J.E., et al., 1979, J. Clin. Invest. 64:1642-1651) on gentamicin-induced acute renal failure. DMTU provided a marked protective effect (confirming the results described above), with both BUN and plasma creatinine significantly lower than those of rats treated with gentamicin alone (Fig. 1). Also, in rats concurrently treated with DMSO, both BUN and plasma creatinine levels were significantly lower than in rats treated with gentamicin alone (Fig. 1).

### 6.2.3. SODIUM BENZOATE OR DEFEROXAMINE HAVE A PROTECTIVE EFFECT AGAINST GENTAMICIN-INDUCED ACUTE RENAL FAILURE

In a third experiment, we examined the effect of a third hydroxyl radical scavenger, sodium benzoate (Till, G.O., et al., 1985, A.J.P. 119(3): 376-384). In addition, we again examined the effect of the iron chelator deferoxamine on gentamicin-induced acute renal failure. The BUN and plasma creatinine were significantly lower in rats that received

sodium benzoate or deferoxamine in addition to gentamicin (Fig. 2). In the same experiment, iron-saturated deferoxamine was only partially protective (BUN of 64±6, n=6).

### 6.2.4. 2,3-DIHYDROXYBENZOIC ACID HAS A PROTECTIVE EFFECT AGAINST GENTAMICIN-INDUCED ACUTE RENAL FAILURE

We also examined the effect of the iron chelator 2,3-dihydroxybenzoic acid (DHB) on gentamicin-induced acute renal failure. DHB exhibited a marked protective effect, with BUN significantly lower than that in rats treated with gentamicin alone (Fig. 3).

### 6.2.5. AN HYDROXYL RADICAL SCAVENGER OR DEFEROXAMINE HAVE A PROTECTIVE EFFECT AGAINST GENTAMICIN-INDUCED RENAL TUBULAR DAMAGE

We examined the histological changes in kidney tissue among the differently treated rats at the end of the experiments described in Sections 6.2.2. and 6.2.3., supra. The histological changes were graded as described in Section 6.1.4., supra, and the results are shown in Table III.

TABLE III

SEMIQUANTITATIVE ANALYSIS OF RENAL HISTOLOGY[1]

| | GRADE | | | | |
|---|---|---|---|---|---|
| GROUP | 0 | 1+ | 2+ | 3+ | 4+ |
| Controls | 14 | - | - | - | - |
| Gentamicin | - | - | 4 | 4 | 6 |
| Gentamicin + DMTU | 2 | 1 | 4 | 1 | - |
| Gentamicin + DMSO | 8 | - | - | - | - |
| Gentamicin + Benzoate | 4 | - | 1 | 1 | - |
| Gentamicin + DFO | 6 | - | - | - | - |

[1]Histologic grading was as follows: 0 = normal; 1 = Areas of focal granulovacuolar epithelial cell degeneration with less than 1% of total tubule population showing epithelial cell desquamation; 2 = Tubular epithelial necrosis and desquamation involving less than 50% of cortical tubules; 3 = Greater than 50% of proximal tubules showing desquamation (uninvolved tubules easily found); 4 = Complete or almost complete proximal tubular necrosis. Numerous cytoplasmic PAS-positive bodies were present in the proximal tubule epithelial cells of all gentamicin-treated animals, with and without interventional treatment. DMTU: dimethylthiourea; DMSO: dimethyl sulfoxide; Benzoate: sodium benzoate; DFO: deferoxamine.

In rats treated with gentamicin alone for eight days, the extent of tubular necrosis varied from less than 10% (Grade 2) to greater than 75% (Grades 3 and 4) of tubules, as described in previous studies (Houghton, D.C., et al., 1978, Am. J. Pathol. 93:137-152). In addition, numerous PAS-positive cytoplasmic bodies (cytosegresomes by electron microscopy) were seen in all of the animals, in agreement with previous studies that have shown the characteristic development of cytosegresomes within 48 hours following the first gentamicin injection (Humes, H.D. and Weinberg, J.M., 1986, in The Kidney, Brenner, B.M. and F.C. Rector, Jr., eds., W.B. Saunders Company, Philadelphia, Pa., pp. 1491-1532). (PAS-positive indicates a reaction with the periodic acid-Schiff reagent to produce an insoluble purple or magenta color visible under the light microscope.) In rats treated with the hydroxyl radical scavengers or with deferoxamine (in addition to gentamicin), the PAS-positive cytoplasmic bodies were also seen in all the animals; however, as shown in Table III, there was a marked reduction in the extent of tubular damage. Light microscopic sections demonstrating the protective effect of deferoxamine on kidneys of gentamicin-treated rats are shown in Figures 4A and 4B.

The data thus indicate that hydroxyl radical scavengers DMTU, DMSO, and sodium benzoate, and the iron chelators deferoxamine and 2,3-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid afford both functional and histological protection against gentamicin-induced acute renal failure in rats.

While not bound to a particular theory, several mechanisms may explain the essential role of iron in mediation of renal damage. The protective effect of deferoxamine may be caused by the prevention of hydroxyl radical formation from hydrogen peroxide in the iron-catalyzed Haber Weiss reaction

(Hoe, S., et al., 1982, Chem.-Biol. Interactions 41:75-81; Aust, S.D., et al., 1985, J. Free Radicals Biology and Medicine 1:3-25):

$$Fe^{3} + O_2^- \dashrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \dashrightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

Alternatively, the requirement for iron could stem from its involvement in the formation of the perferryl ion, a reaction which requires iron, NADPH-cytochrome P-450 reductase, NADPH, and oxygen:

$$Fe^{2+} + O_2 \dashrightarrow Fe^{2+}O_2 \text{ (perferryl ion)} \rightleftharpoons Fe^{3+}O_2^-$$

This explanation presumes that $Fe^{3+}O_2^-$ is the free radical or precursor thereof which mediates renal toxicity. The protective effect of several hydroxyl radical scavengers would seem to favor the role of iron via the Haber Weiss reaction.

We did not examine the biological processes that may be affected by the hydroxyl radical, that lead to acute renal failure. One of the mechanisms by which the hydroxyl radical has been postulated to cause tissue damage is by causing peroxidation of membane lipids. Lipid peroxidation has been shown to be associated with tissue injuries in which the hydroxyl radical has been implicated as being important (Ward, P.A., et al., 1985, J. Clin. Invest. 76:517-527; Till, G.O., et al., 1985, A.J.P. 119(3): 376-384). However, based on the time course, the lipid peroxidation appears to be a consequence of oxygen-mediated tissue injury rather than a mediator or propagator thereof (Till, G.O., et al., supra). Similarly, in gentamicin-treated rats there is an increase in the content in

the kidney cortex of malondialdehyde (TBA-reactive material), a lipid peroxidation product, but preventing lipid peroxidation does not prevent the gentamicin-induced acute renal failure (Ramsammy, L.S., et al., 1986, J. Pharm. Exp. Therap. 238(1):83-86). Based on these observations, it is likely that the role of the hydroxyl radical in gentamicin-induced acute renal failure is likely to be its effects on biological processes other than lipid peroxidation.

WHAT IS CLAIMED IS:

1. A method for preventing or reducing toxicity of an aminoglycoside comprising administering in vivo an effective dose of a compound which prevents the generation of, effectively scavenges, or detoxifies a reactive oxygen metabolite which mediates a nephrotoxic effect of the aminoglycoside.

2. The method according to claim 1 in which the compound comprises a free radical scavenger.

3. The method according to claim 2 in which the free radical scavenger comprises a hydroxyl radical scavenger.

4. The method according to claim 3 in which the hydroxyl radical scavenger comprises dimethylthiourea.

5. The method according to claim 3 in which the hydroxyl radical scavenger comprises dimethyl sulfoxide.

6. The method according to claim 3 in which the hydroxyl radical scavenger comprises sodium benzoate.

7. The method according to claim 1 in which the compound comprises an iron-chelating agent.

8. The method according to claim 7 in which the iron-chelating agent comprises deferoxamine.

9. The method according to claim 7 in which the iron-chelating agent comprises 2,3-dihydroxybenzoic acid.

10. The method according to claim 1 in which the compound comprises an enzyme.

11. The method according to claim 10 in which the enzyme comprises superoxide dismutase.

12. The method according to claim 10 in which the enzyme comprises catalase.

13. The method according to claim 1 in which the compound comprises an oxidizable compound which undergoes oxidation by the reactive oxygen metabolite.

14. The method according to claim 1 in which the compound comprises a molecule which increases the effective in vivo concentration of an endogenous agent that prevents the generation of, effectively scavenges, or detoxifies a reactive oxygen metabolite which mediates a nephrotoxic effect of the aminoglycoside.

15. The method according to claim 14 in which the endogenous agent comprises an oxidizable agent which undergoes oxidation by the reactive oxygen metabolite.

16. The method according to claim 15 in which the compound comprises a biosynthetic precursor of the oxidizable agent.

17. The method according to claim 16 in which the oxidizable agent comprises glutathione.

18. The method according to claim 17 in which the synthetic precursor is selected from the group consisting of gamma-glutamylcysteine, gamma-glutamylcystine, and gamma-glutamylcysteine disulfide.

19. The method according to claims 1, 2, 3, 7, 10, 13 or 16 in which the antibiotic comprises gentamicin.

20. The method according to claim 8 in which the antibiotic comprises gentamicin.

21. The method according to claim 1 or 20 in which the administration is intramuscular.

22. A drug composition of reduced toxicity comprising a mixture of

(a) an effective amount of an aminoglycoside; and

(b) an effective amount of a compound which prevents the generation of, effectively scavenges, or detoxifies a reactive oxygen metabolite and prevents or reduces a nephrotoxic effect of the aminoglycoside.

23. The drug composition of claim 22 in which the compound comprises a free radical scavenger.

24. The drug composition of claim 23 in which the free radical scavenger comprises a hydroxyl radical scavenger.

25. The drug composition of claim 24 in which the hydroxyl radical scavenger comprises dimethylthiourea.

26. The drug composition of claim 24 in which the hydroxyl radical scavenger comprises dimethyl sulfoxide.

27. The drug composition of claim 24 in which the hydroxyl radical scavenger comprises sodium benzoate.

28. The drug composition of claim 22 in which the compound comprises an iron chelating agent.

29. The drug composition of claim 28 in which the iron chelating agent comprises deferoxamine.

30. The drug composition of claim 28 in which the iron chelating agent comprises 2,3-dihydroxybenzoic acid.

31. The drug composition of claim 22 in which the compound comprises an enzyme.

32. The drug composition of claim 31 in which the enzyme comprises superoxide dismutase.

33. The drug composition of claim 31 in which the enzyme comprises catalase.

34. The drug composition of claim 22 in which the compound comprises an oxidizable compound which undergoes oxidation by the reactive oxygen metabolite.

35. The drug composition of claim 22 in which the compound comprises a molecule which increases the effective in vivo concentration of an endogenous agent that prevents the

generation of, effectively scavenges, or detoxifies a reactive oxygen metabolite which mediates a nephrotoxic effect of the aminoglycoside.

36. The drug composition of claim 35 in which the endogenous agent comprises an oxidizable compound which undergoes oxidation by the reactive oxygen metabolite.

37. The drug composition of claim 36 in which the compound comprises a biosynthetic precursor of the oxidizable agent.

38. The drug composition of claim 37 in which the oxidizable agent comprises glutathione.

39. The drug composition of claim 38 in which the biosynthetic precursor is selected from the group consisting of gamma-glutamylcysteine, gamma-glutamylcystine, and gamma-glutamylcysteine disulfide.

40. The drug composition of claim 22, 23, 24, 28, 31 or 34, or 37 in which the antibiotic comprises gentamicin.

41. The drug composition of claim 29 in which the antibiotic comprises gentamicin.

42. The drug composition of claim 22 or 41 which is administered intramuscularly.

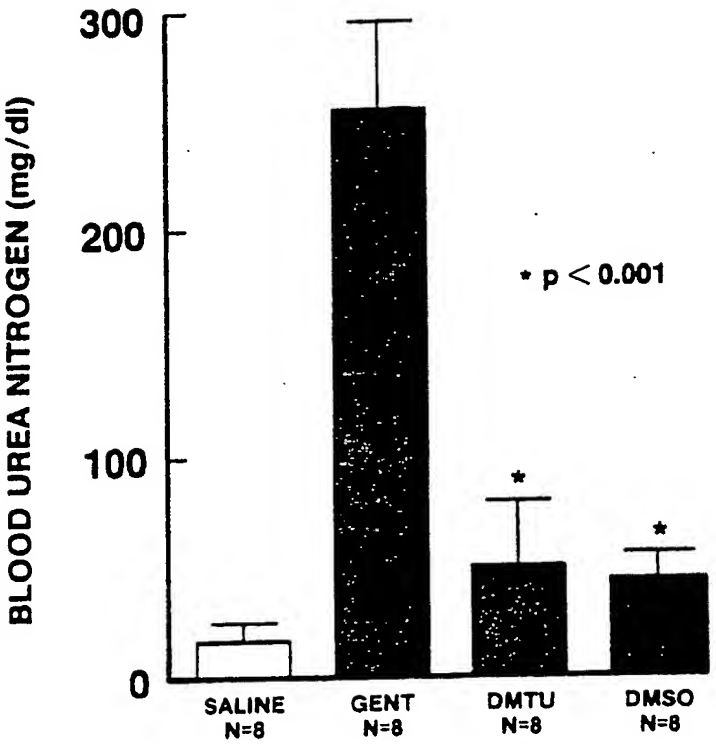
BLOOD UREA NITROGEN (mg/dl)
300
200
100
0
* p < 0.001
*
*
SALINE N=8
GENT N=8
DMTU N=8
DMSO N=8

FIG. 1 A

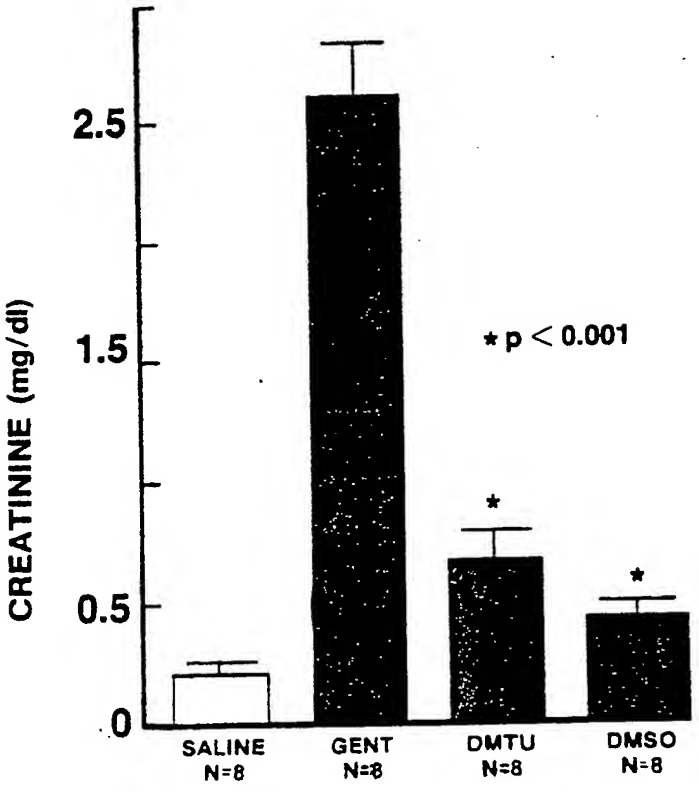
CREATININE (mg/dl)
2.5
1.5
0.5
0
* p < 0.001
*
*
SALINE N=8
GENT N=8
DMTU N=8
DMSO N=8

FIG. 1 B

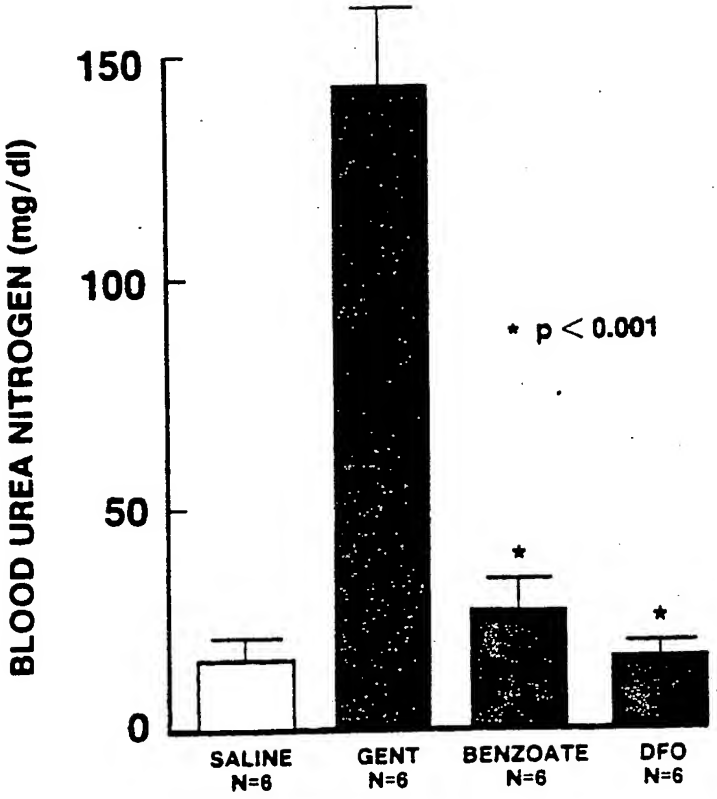
BLOOD UREA NITROGEN (mg/dl)
150
100
50
0
* p < 0.001
*
*
SALINE N=6
GENT N=6
BENZOATE N=6
DFO N=6

FIG. 2 A

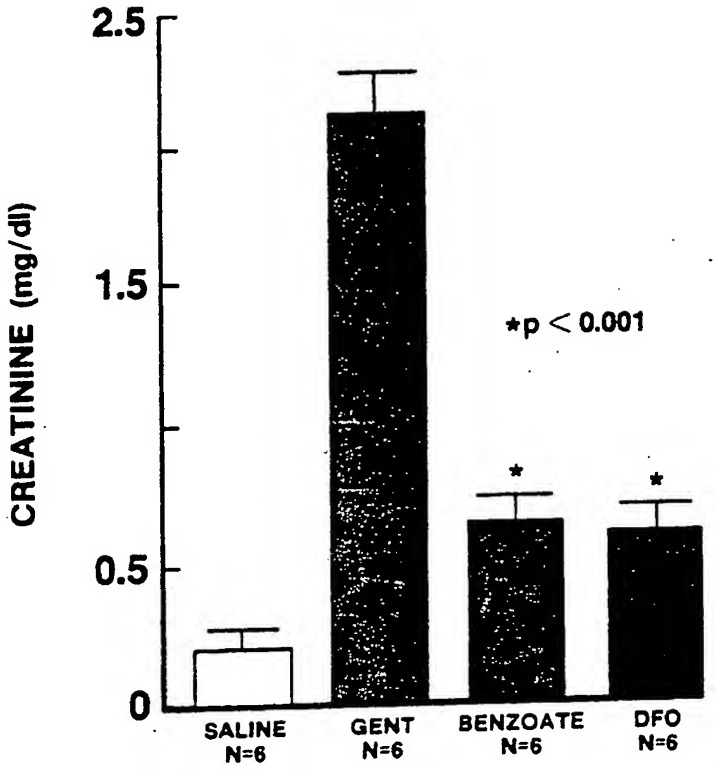
2.5
1.5
0.5
0
CREATININE (mg/dl)
*p < 0.001
*
*
SALINE N=6
GENT N=6
BENZOATE N=6
DFO N=6

FIG. 2B

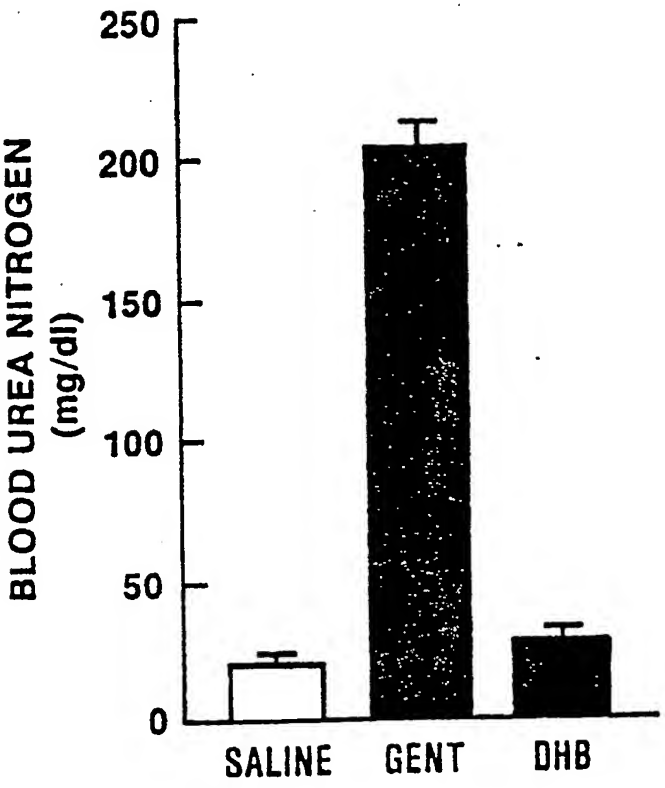


FIG. 3

FIG. 4A

FIG. 4B

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/03468

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K 31/17, 31/70

US CL : 424/10; 514/37,39,41,580,922,936

## II. FIELDS SEARCHED

Minimum Documentation Searched [4]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 424/10; 514/37,39,41,580,922,936 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| X<br>Y | Chemical Abstracts, Volume 95, No. 1, issued 6 July 1981 (Columbus, Ohio, USA), PIERSON ET AL., "Prophylaxis of Kanamycin-induced ototoxicity by a radioprotectant", see page 459, column 1, the Abstract No. 463q, HEAR. RES. 1981, 4(1), 79-87 (Eng). | 1,2 and 13<br>1-4,6-21, 23-25 and 27-42 |
| X | EP, 0,002,835 A1 (SHIONOGI & CO., LTD) 11.07.79, see page 10, lines 25-26 and line 37. | 22-24,27, and 42 |
| X | Chemical Abstracts, Volume 92, No. 13, issued 31 March 1980 (Columbus, Ohio, USA), RUBINSTEIN ET AL., "The effect of Dimethyl Sulfoxide on tissue distribution of Gentamicin", see page 20, column 1, the Abstract No. 104039K, Experientia 1980, 36(1), 92-3(Eng). | 5,22,26 |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search [2] | Date of Mailing of this International Search Report [2] |
|---|---|
| 27 January 1988 | 17 MAR 1988 |
| **International Searching Authority [1]** | **Signature of Authorized Officer [20]** |
| ISA/US | Richard Kearse<br>Richard Kearse |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No ¹⁸ |
|---|---|---|
| Y | Chemical Abstracts, Volume 99, No. 11, issued 12 September 1983 (Columbus, Ohio, USA), ZUNINO ET AL., "Protective effect of reduced glutathione against cis-dichlorodiammine platinum(II)-induced nephrotoxicity and lethal toxicity", see page 27, column 2, the Abstract No. 82122X, Tumori 1983, 69(2), 105-11 (Eng). | 1-4,6-21, 23-25, and 27-42 |
| Y | Chemical Abstracts, Volume 90, No. 5, 29 January 1979, (Columbus, Ohio, USA), McGINNESS ET AL., "Amelioration of cis-platinum nephrotoxicity by orgotein (superoxide dismutase)", see page 44, column 2, the Abstract No. 34001z, Physiol. Chem. Phys. 1978, 10(3), 267-77, (Eng). | 1-4,6-21, 23-25 and 27-42 |
| Y | Chemical Abstracts, Volume 95, No. 25, issued 21 December 1981 (Columbus, Ohio USA), Graziano et al., "The effect of heavy metal chelators on the renal accumulation of platinum after cis-dichlorodiammine platinum II administration to the rat", see page 32, column 2, the Abstract No. 214975g, Br. J. Pharmacol. 1981, 73(3), 649-54 (Eng). | 1-4,6-21 23-25 and 27-42 |
| Y | Chemical Abstracts, Volume 96, No. 9, issued 1 March 1982 (Columbus, Ohio, USA), Ishizawa et al., "Protection by sodium thiosulfate and thiourea against lethal toxicity of cis-diamminedichloro-platinum II in bacteria and mice", see page 32, column 2, the Abstract 62689X, JPN. J. Pharmacol. 1981, 31(6), 883-9 (Eng). | 1-4,6-21 23-25 and 27-42 |
| Y | Chemical Abstracts, Volume 98, No. 15, issued 11 April 1983 (Columbus, Ohio, USA), McGINNESS ET AL., "An in vivo enzymic probe for superoxide and peroxide production by chemotherapeutic agents", see page 34, column 1, the Abstract No. 119263b, Pathol. Oxygen 1982, 191-206 (Eng). | 1-4,6-21 23-25 and 27-42 |
| X<br>Y | SUMPIO ET AL., "Reduction of the Drug-Induced nephrotoxicity by ATP-$MgCl_2$" J. of Surg. Res. 38(5), 429-437 (1985) (Eng), see page 435, column 2, lines 23-25. | 22 and 23<br>1-4,6-21, 24-25 and 27-42 |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

| | | |
|---|---|---|
| X<br>Y | SUMPIO ET AL., "Reduction of the Drug-Induced Nephrotoxicity by ATP-$MgCl_2$", J. of Surg. Res. 38(5), 438-445 (1985), (Eng) see the entire document. | 22 and 23<br>1-4,6-21, 24-25 and 27-42 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.